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RAS testing in metastatic colorectal cancer: advances in Europe

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Received: 4 June 2015 / Revised: 20 October 2015 / Accepted: 22 October 2015 / Published online: 16 November 2015
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Abstract Personalized medicine shows promise for maximizing efficacy and minimizing toxicity of anti-cancer treatment. *KRAS* exon 2 mutations are predictive of resistance to epidermal growth factor receptor-directed monoclonal antibodies in patients with metastatic colorectal cancer. Recent studies have shown that broader *RAS* testing (*KRAS* and *NRAS*) is needed to select patients for treatment. While Sanger sequencing is still used, approaches based on various methodologies are available. Few CE-approved kits, however, detect the full spectrum of *RAS* mutations. More recently, “next-generation” sequencing has been developed for research use, including parallel semiconductor sequencing and reversible termination. These techniques have high technical sensitivities for detecting mutations, although the ideal threshold is currently unknown. Finally, liquid biopsy has the potential to become an additional tool to assess tumor-derived DNA. For accurate and timely *RAS* testing, appropriate sampling and prompt delivery of material is critical. Processes to ensure efficient turnaround from sample request to *RAS* evaluation must be implemented so that patients

receive the most appropriate treatment. Given the variety of methodologies, external quality assurance programs are important to ensure a high standard of *RAS* testing. Here, we review technical and practical aspects of *RAS* testing for pathologists working with metastatic colorectal cancer tumor samples. The extension of markers from *KRAS* to *RAS* testing is the new paradigm for biomarker testing in colorectal cancer.

Keywords Colorectal cancer · *RAS* · Biomarkers · Sequencing · Quality assurance programs

Introduction

Personalized medicine and tailoring of therapy to individual patients is a promising approach for maximizing efficacy and minimizing the toxicity of anti-cancer treatment [1]. Furthermore, such an approach can generate healthcare cost savings, as treatments are given only to patients likely to benefit [2]. In recent years, a few molecular tumor biomarkers—characteristics “that can be objectively measured and evaluated as an indicator of pathogenic processes or treatment response” [3]—have been identified, enabling anti-cancer treatments to be better tailored to individual patients’ tumors [1]. Those molecular biomarkers have provided alternative therapeutic options and improved patient outcomes, especially in metastatic colorectal cancer (mCRC). In the European Union, there were an estimated 342,000 new cases of colorectal cancer in 2012 (46.3 per 100,000 individuals), and mCRC remains the second leading cause of cancer-related deaths (215,000 deaths in 2012) [4].

Epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies (mAbs) have been investigated for the treatment of mCRC, but the benefits to patients were small in initial clinical trials when these agents were unselectively added to standard care. Subsequently, *KRAS* mutations,

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particularly those in exon 2 (detectable in about 30–40 % of patients with mCRC) [5, 6] were identified as a predictive biomarker of resistance to the EGFR-targeted antibodies. These mutations result in constitutive activation of the GTPase *KRAS*, leading to activation of signaling downstream of the EGFR [7–10]. Consequently, in patients with exon 2 wild-type (WT) *KRAS* tumors, addition of EGFR inhibitory antibodies to standard chemotherapy significantly improved progression-free survival (PFS) versus chemotherapy alone [8, 11]. For example, in the phase III, first-line PRIME study, oxaliplatin, 5-fluorouracil (5-FU), and leucovorin (FOLFOX4) plus panitumumab was associated with a median PFS of 9.6 versus 8.0 months for FOLFOX4 alone ($p = 0.02$), while median overall survival [OS] was 23.9 and 19.7 months, respectively ($p = 0.072$) [8]. Similarly, median PFS with irinotecan, 5-FU, and leucovorin (FOLFIRI) plus cetuximab in the CRYSTAL study was 9.9 versus 8.4 months with FOLFIRI alone ($p = 0.0012$) and OS was 23.5 versus 20.0 months ($p = 0.0093$), respectively [11]. In patients with mutant *KRAS* tumors, combination of anti-EGFR therapy with irinotecan-based chemotherapy is associated with little or no benefit [9, 12, 13], while combination of anti-EGFR therapy with oxaliplatin-based chemotherapy in such patients may be detrimental to both PFS and OS [8, 14, 15].

While *KRAS* exon 2 mutations are undoubtedly useful for predicting a lack of activity of EGFR-targeted mAbs, use of this biomarker increased response rates only from ~15 % in an unselected population to ~30 % in patients with *KRAS* exon 2 WT mCRC [16]. Analyses of tumor samples from the PRIME study in mCRC have shown that more comprehensive *RAS* testing (i.e., exon 2, 3, and 4 of both *KRAS* and *NRAS*) better selects those patients more likely to respond to EGFR inhibitors, with *RAS* WT populations experiencing a statistically significant improvement in OS versus chemotherapy alone (26 versus 20.2 months; $p = 0.04$) [14]. Furthermore, data from the phase II PEAK and phase III FIRE-3 studies indicated that patients with WT *RAS* tumors experienced greater benefit from mFOLFOX6 plus panitumumab (PEAK) or FOLFIRI plus cetuximab (FIRE-3) than from mFOLFOX6 or FOLFIRI, respectively, plus bevacizumab in terms of PFS in PEAK only (PEAK, 13.0 versus 9.5 months; $p = 0.029$ and FIRE-3, 10.0 versus 10.3 months; $p = 0.55$) and OS in both (PEAK, 34.2 versus 24.3 months; $p = 0.009$ and FIRE-3, 28.7 versus 25.0 months; $p = 0.017$) [17, 18]. Taken together, mutations in either *KRAS* or the closely related *NRAS* gene, but not in the *BRAF* gene, were found to be associated with lack of response to EGFR-targeted mAbs [18–24].

In several large studies including patients with mCRC, over half of patients were found to have tumors harboring mutations in *KRAS* or *NRAS* (Table 1) [10, 14, 18, 19, 25–31]. For example, 17 % of patients with tumors WT for *KRAS* codons 12 and 13 in the PRIME study (~10 % of patients overall) were found to have mutations in other *KRAS* codons and/or

NRAS [14]. A pooled analysis of five studies ($n = 2832$ patients with *KRAS* and *NRAS* data available) showed that the prevalence of *RAS* mutations was 55.9 % (95 % confidence interval [CI], 53.9–57.9 %) [32]. More recently, a meta-analysis of nine randomized controlled trials ($n = 5948$) showed that EGFR-targeted mAb therapy in a *RAS* WT population had a significantly superior PFS ($p < 0.001$) and OS ($p = 0.008$) treatment effect compared with in a *RAS* mutant population [33].

As patients with mutant *RAS* tumors do not benefit from EGFR inhibitors, and may even experience worse outcomes when EGFR inhibitors are combined with oxaliplatin-based chemotherapy [14, 15], the European Public Assessment Report summaries of product characteristics (SmPCs) for panitumumab and cetuximab specify that “evidence of wild-type *RAS* (*KRAS* and *NRAS*) status is required before initiating treatment” [34, 35]. *RAS* gene testing has therefore become an important part of the work-up of patients with mCRC in Europe. Thus, it is essential that genetic testing is conducted to a high standard and as quickly as possible in case a patient is eligible for EGFR-targeted treatment. The highest quality of testing is achieved when the genetic analysis is carried out in the context of a histopathological evaluation [36, 37] to ensure that appropriate tissue areas are included in the analysis. To test and ensure the quality of *RAS* testing (i.e., the correct identification of WT [specificity] and mutant *RAS* [sensitivity] to eliminate false-positive and false-negative results), external quality assurance (EQA) programs play a critical role. This view is supported by the European Medicines Agency (EMA), as the SmPCs for panitumumab and cetuximab state that “mutational status should be determined by an experienced laboratory using validated test methods for detection of *KRAS* and *NRAS* (exons 2, 3, and 4) mutations” [34, 35]. As “experience” is only vaguely definable, a test by an EQA program is a suitably objective tool to verify a laboratory’s experience as noted in the SmPC for panitumumab: “if [panitumumab] is to be used in combination with FOLFOX then it is recommended that mutational status be determined by a laboratory that participates in a *RAS* EQA program or WT status be confirmed in a duplicate test” [34].

Full tumor *RAS* testing—rather than solely *KRAS* exon 2 testing—is included in treatment guidelines for mCRC such as those from the European Society for Medical Oncology (ESMO), the National Comprehensive Cancer Network (NCCN), the German Cancer Society Association of Medical Oncology (AIO), and the Dutch Landelijke Werkgroep Gastro Intestinale Tumoren [38–41].

The shift from screening a single *KRAS* locus to multiple *RAS* loci highlights the evolution of biomarker testing to detect multiple mutation sites. This change in approach may affect the choice of screening methods used, as many different techniques for assessing *RAS* mutation status are now available [42]. The aim of this review is to describe these different

Table 1 Prevalence of *RAS* mutations in patients with metastatic colorectal carcinoma

Study	Detection method	RAS mutation prevalence						
		KRAS			NRAS			Total
		Exon 2	Exon 3	Exon 4	Exon 2	Exon 3	Exon 4	
PRIME [14]	TheraScreen	40 %	4 % ^a	6 %	3 %	4 % ^a	0 %	52 % ^a
20050408 [19]	DxS/Qiagen/NGS/Sanger	42 %	2 % ^a	NS	5 % ^a		NS	NS
20050408 (updated) [26]	NGS/Sanger + WAVE/SURVEYOR®	43 %	5 % ^a	5 %	4 %	3 % ^a	1 %	NS
20050181 [25]	DxS/Qiagen/NGS/Sanger	45 %	4 %	8 %	2 %	6 %	0 %	58 %
PICCOLO [27]	Pyrosequencing	NA ^b	NA ^b	4 % ^c	6 % ^a		NS	NS
PEAK [18]	Therascreen + WAVE/SURVEYOR®	NA ^d	4 %	7 %	5 %	6 %	0 %	NS
CRYSTAL [10]	LightMix	36 %	NS	NS	NS	NS	NS	NS
CRYSTAL (updated) [28]	BEAMing	37 %	3 %	5 %	4 %	3 %	2 %	43 %
COIN [29]	Pyrosequencing	43 % ^a		NS	4 % ^{a,e}		NS	NS
FIRE-3 [30]	Pyrosequencing	NA ^d	4 %	5 %	4 %	2 %	0 %	NS
OPUS [31]	BEAMing	NA ^d	6 %	9 %	7 %	5 %	1 %	NS

BEAM beads, emulsions, amplification, and magnetics, *NGS* next-generation sequencing, *NS* not specified, *PCR* polymerase chain reaction

^a Not including codon 59

^b Study population limited to patients with *KRAS* (exon 2 and exon 3 codon 61) wild-type tumors

^c Not including codon 117

^d Study population limited to patients with *KRAS* (exon 2) wild-type tumors

^e Not including codon 13

methods, focusing on those most commonly used in Europe: sequencing (Sanger sequencing, pyrosequencing, next-generation sequencing [NGS] techniques), other in vitro diagnostic techniques, and laboratory-developed techniques. These approaches differ in both their limit of detection (LOD; sensitivity) and their specificity. Some of the key issues in *RAS* testing will be discussed, including sensitivity of testing and EQA programs.

Initial material—DNA from formalin-fixed, paraffin-embedded tissue blocks

To assess *RAS* mutation status, analysis is usually performed on DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue blocks [43]. As a result of chemical modification and DNA fragmentation, the amplicon size in polymerase chain reactions (PCR) is limited to about 150 base pairs, when sequencing is performed on FFPE-derived DNA [44]. Moreover, for NGS, the initial procedure for DNA isolation from FFPE tissue blocks remains the same as for conventional DNA sequencing [43] and is, therefore, subject to some of the same issues of DNA quantity and quality.

DNA sequencing methods

Most sequencing methods can screen a gene locus for mutations. Newer sequencing methods provide quantitative

information and increase the number of loci that can be analyzed in parallel.

Sanger sequencing (also known as “terminated chain sequencing” or “dideoxy sequencing”) is considered to be a standard method for analyzing DNA sequences and thus also for *RAS* testing [43, 44]. Overall, it is the most widely used method of *RAS* testing in Europe [45], although its use varies from country to country. Major improvements to the original Sanger technique have resulted in reduced reagent volumes and consumable costs, as well as increased throughput [46], although it is still considered time consuming compared with some other methods [44]. The advantage of this method is its ability to detect all types of mutation (single-nucleotide substitutions, insertions, and deletions) in the amplified locus. If DNA is isolated from FFPE tissue, the size of the amplicons to be analyzed should not exceed 150 base pairs. To ensure reproducibility, bidirectional sequencing reactions are performed using forward and reverse primers [47]. Each run gives the nucleotide sequence of one amplified locus; up to 96 runs can be performed in parallel (usually 16 to 24). In addition, the LOD for Sanger sequencing is relatively modest compared with other techniques. Multiple studies have shown that Sanger sequencing requires at least 10–25 % of the neoplastic cells in the sample to contain *KRAS* mutations for reliable detection [48]. Several enrichment methods are available, however, to increase the concentration of mutant DNA, thus improving the overall sensitivity of the detection process

(Table 2) [49–53]. Another limitation of Sanger sequencing is the low level of precision in the variant fraction (i.e., the percentage of tested alleles that are mutant); the error rate (i.e., the percentage of incorrectly identified bases) of the Sanger sequencing reaction is estimated at 0.001 to >1 %, depending on the software that is used post processing [54].

Pyrosequencing is an alternative method to Sanger sequencing that can provide both quantitative information and a low technical LOD (5–10 %) [55], although the sequencing error rate may be higher (4–25 %) [56]. During pyrosequencing, the incorporation of each nucleotide is followed in real time by the emission of a specific light unit based on the amount of pyrophosphate, which is released in stoichiometric amounts during the elongation reaction and converted by an enzymatic cascade into adenosine triphosphate (ATP) molecules each time a nucleotide is incorporated. The ATP serves as the energy donor in a bioluminescence reaction resulting in emission of light that can be measured as a peak in the pyrogram output. Pyrosequencing allows the local separation of mutant and WT signals, although the presence of homopolymers can limit the method, as linearity is present only for some nucleotides. In this approach, each run gives the nucleotide sequence of one amplified locus, but the size of the sequence (less than 20 base pairs) is often shorter than those produced via Sanger sequencing. Up to 96 runs can be performed in parallel.

Next-generation DNA sequencing

NGS platforms involve massively parallel sequencing of bulks of clonally amplified DNA molecules that are spatially separated and have helped to shift the focus of sequence analysis from a few loci to multiple genes and loci. With NGS, each run can give the nucleotide sequence for several loci and several tumor samples. Importantly, NGS methods can detect any variant from single-nucleotide changes to insertions or deletions, and even translocations, in a single multiplex PCR reaction. Depending on the depth of sequencing, the LOD of NGS is about 1–5 %. Results are provided as quantitative information. The most sensitive part of the NGS is the bioinformatic pipeline generating Binary Sequence Alignment Map/Sequence Alignment Map format (BAM/SAM) files. Specific statistical algorithms are used to score the quality of a single read by aligning the sequence to a reference genome. As a consequence, some readouts do not detect all of the deletions, insertions, or other mutations that are detectable with Sanger sequencing, as some mutations are found by the basic algorithms but subsequently excluded and, therefore, do not occur in the resulting variant caller files. Therefore, extensive validation of the entire procedure is essential, as is the use of internal control to assess the reproducibility of the runs.

With the increasing number of biomarkers, NGS allows screening of a large number of patients and markers with a fixed and limited amount of DNA. For NGS using Ion Torrent technology, as little as 1 ng input material is needed, whereas conventional sequencing technologies need 10 to 20 ng per run and marker [57]. As a first step, a DNA library is generated by amplification of the DNA fragments to be sequenced (the gene panel) in a single reaction (sometimes in up to five different tubes) or capture hybridization followed by the amplification of this pre-library either by emulsion PCR (emPCR; e.g., on spheres) or by bridge PCR on solid flow cells [46]. As capture hybridization needs much more input material compared with the panel-amplification approach, the latter is mostly used in the analysis of biomarkers. Ultimately, a single NGS run may comprise many millions or even billions of spatially distributed, clonally amplified amplicons [58]. Custom assays can be developed (AmpliSeq™ for Ion Torrent, GeneRead for Qiagen, TruSeq™ for Illumina) using amplicons designed with a starting size of 150 bp, optimal for FFPE samples. Finally, the quality of the sample can be extremely limited, and strategies have been proposed to identify samples with low probability of success (e.g., the Illumina FFPE QC Kit based on qPCR validation) before any NGS run. This highlights that not all samples are suitable for analysis on these platforms.

Historically, three principal NGS technologies have been commercially available [58]. The first of these, sequencing-by-ligation, no longer has any clinical application. The second is sequencing-by-synthesis [59], and the third is based on the

Table 2 Methods for enriching mutant DNA for *RAS* testing [49–53]

Method	Features
Microdissection	Allows precise analysis of tumor tissue by reducing contamination with normal tissue and, therefore, improving sensitivity for <i>RAS</i> mutations present in the neoplastic cell sample
COLD PCR	Exploits the critical temperature at which mutation-containing DNA is preferentially melted over WT (sensitivity, 0.01–10 %)
Allele-specific amplification	Uses primers designed with a 3' terminal nucleotide that pairs with the mutant sequence but not with the WT (e.g., the amplification refractory mutation system) (sensitivity, 0.1–1 %)
Blocking amplification of WT sequence	For example, by binding high-affinity peptide nucleic acid probes (PCR clamp; sensitivity in routine applications, 0.1–1 %)
Selective destruction of WT samples	Exploiting restriction enzyme sites in the WT sequence (e.g., restriction endonuclease-mediated selective PCR and simultaneous PCR/restriction fragment length polymorphism) (sensitivity, 0.001–0.1 %)

COLD co-amplification at lower denaturation temperatures, *PCR* polymerase chain reaction, *WT* wild-type

pyrosequencing approach. As an alternative to pyrophosphate measurement, hydrogen ions/protons can be used as a hydrogen ion is released for each nucleotide incorporated. These can be measured as an induced current peak on a negatively charged semiconductor, which is the basis of the detection unit or chip. Currently available NGS platforms are summarized in Table 3. [60–62] Of these, the Illumina MiSeq and Life Technologies' Ion Torrent Personal Genome Machine™ (PGM™) are the most commonly used in Europe (H. Van Krieken, personal communication), and NGS sequencing methods are expected to replace older methods completely in the coming years.

MiSeq (Illumina Sequencing Systems)

MiSeq is based on a sequencing-by-synthesis approach [59], using fluorescently labeled reversible-terminator nucleotides and clonally amplified DNA templates on acrylamide-coated glass flow cells [63]. Originally, MiSeq was developed as a modification of the earlier Genome Analyzer and HiSeq machines and was designed for lower throughput and fast-turnaround appropriate for smaller laboratories and clinical diagnostics. The MiSeqDx is a special type of MiSeq developed for clinical analysis [63, 64]. The error rate of the Illumina technology is estimated to be <0.4 % [63, 65]. More recently, Illumina has introduced the NextSeq system, which is broadly similar to MiSeq, although the run time is faster (12–30 versus 5–55 h for MiSeq) [61].

Ion Torrent (Life Technologies)

The Ion Torrent PGM™ sequencer is based on ion semiconductor technology [66]. DNA fragments are clonally amplified by emulsion PCR on the surface of 3-μm diameter beads, which are then loaded into wells on a proton-sensing silicon wafer. PGM™ was the first commercial sequencing machine that did not require fluorescence and camera scanning, resulting in higher speed, lower cost, and smaller instrument size [64]. The error rate with Ion Torrent sequencing has been estimated at 1.8–1.9 % [63, 65], mostly because of higher error rates in the detection of homopolymer stretches. Ion Torrent sequencing has been recently used to assess *RAS* status of colorectal carcinoma tumors in the CAPRI-GOIM clinical trial [57], and a

panel of well-known predictive markers in the receptor tyrosine kinase pathway, including *KRAS* and *NRAS*, has been developed by the OncoNetwork Consortium [67].

Hypersensitive methods

For the detection of single mutation events in a background of WT sequences, digital PCR techniques [68] were developed that can achieve LODs of <0.1 % for specific hotspots. If the coverage, and thus the depth, of NGS techniques are increased, they can also reach similar LODs [69]. Sensitivity can also be increased by using single-molecule techniques that discriminate between biological and technical duplicates, such as single-molecule molecular inversion probes [70].

The potential applications for hypersensitive methods include detection of mutations in circulating plasma DNA (the so-called liquid biopsy), monitoring of metastatic disease under therapy, detection of emergence of resistance to targeted agents, analysis of small or poor-quality DNA samples, and as a possible additional parameter alongside histopathology for the staging of tumors [71]. Such approaches have been applied in research and for free plasma DNA detection; there will be no direct application for molecular *RAS* testing in routine clinical practice until the clinical validity of these techniques can be determined. The preparation of such assays is generally easier than for NGS assays, but the scope of the targeted markers is greatly reduced, mostly focusing on a single gene locus or small number of loci.

BEAMing

BEAMing (beads, emulsions, amplification, and magnetics) [72] has been used for *RAS* analysis of samples from clinical trials of EGFR inhibitors [73, 74]. Single DNA molecules are attached to single magnetic beads and are amplified by using emPCR, resulting in beads spiked with thousands of clonally identical PCR products. These are hybridized with mutation-specific probes coupled to different fluorochromes, which are then counted by fluorescence-activated cell sorting (FACS). Overall, BEAMing is more suited to the detection of fewer hotspot variants and has the disadvantage that only limited information can be retrieved, which depends mostly on the amount of available fluorochromes and the filter sets of the

Table 3 Examples of currently available next-generation sequencing platforms

	Principle	Amplification
454 (Roche) [60]	Pyrosequencing	Emulsion PCR
Junior (Roche)	Pyrosequencing	Emulsion PCR
HiSeq/MiSeq (Illumina) [61]	Reversible termination	Bridge PCR
Ion Torrent (Life Technologies) [62]	Semiconductor sequencing	Emulsion PCR

PCR polymerase chain reaction

FACS device. In most cases, up to four different events can be analyzed per run, which limits the width of the detection. A single nucleotide can be analyzed per test (e.g., the nucleotide 34 position in *KRAS*); therefore, for detection of mutations in positions 34, 35, and 38, three BEAMing reactions are necessary. Detection of all three positions (34, 35, and 38) in a single BEAMing reaction can be achieved only by using one fluorochrome each for the WT sequence at nucleotides 34, 35, and 38, and at the expense of knowing the exact type of alteration (e.g., c.34G>A). Although the sensitivity of BEAMing is 0.01 %, it must be emphasized that a cutoff of 5 % mutant alleles in tumor samples has been adopted when this technology has been used in clinical trials [73, 74].

Droplet Digital PCR

Droplet Digital PCR (ddPCR) techniques involve compartmentalization of DNA, again using emPCR. Clonally amplified PCR products of an original single DNA fragment are contained in the micelles of a water-in-oil emulsion [75, 76], together with mutation-specific probes that can be obtained from TaqMan® assays (Applied Biosystems). These droplets are counted directly by flow cytometry. This approach can be multiplexed (up to seven in a single run) so, as with BEAMing, multiple runs are necessary to cover the multiple genetic variations of *KRAS* and *NRAS*. The ddPCR detection unit is, however, easier to work with than the BEAMing unit as the ddPCR products can be measured directly without the additional steps required when using the BEAMing technique. ddPCR techniques in association with liquid biopsies show considerable potential, although their precise clinical role is currently unclear [71].

Approved in vitro diagnostic tools for *RAS* testing

Commercially available kits, instruments, and reagents that are intended by the manufacturer to be used for in vitro examination of patient specimens for the purpose of providing information are governed by European Directive 98/79/EC on in vitro medical devices [77]. Key objectives of this directive are to ensure that in vitro diagnostic medical devices provide a high level of health protection and attain the attributed performance level, and products that conform to this standard carry “CE” marking. The directive does not regulate products for research-use only nor does it cover non-commercialized patient diagnostic tools produced within health institution laboratories for use solely within that environment. It is important to note that the CE mark simply means that the product itself has consistent quality and does not provide any information about specificity and sensitivity in the lab where the kit is used.

The advantage of commercial CE tests is the validation process that they have undergone. Thus, after the in-house

verification of a CE-kit, no additional testing is needed, which is in contrast to non-CE-kits where a change in the batch of materials used requires quality testing of the new material. A list of CE-approved *RAS* tests available in Europe (complete as of December 2014, to the best of our knowledge) is shown in Table 4 [78–92]. Some techniques identify the somatic mutation directly, while others are screening methods that involve a two-step process for positive samples. In addition, other CE-approved kits are available for assessing *KRAS* exon 2 status (with or without exon 3 codon 61), and laboratories may wish to use these, supplemented with laboratory-developed tests to complete the *RAS* analysis.

Of the available CE-approved kits, only five detect mutations in both the *KRAS* and *NRAS* genes: (1) CRC *RAS*Scan™ (SURVEYOR®/WAVE®), (2) CRC *RAS*seq™ (both Transgenomics, Inc.), (3) Therascreen® *KRAS* and *NRAS* Pyro® kits (Qiagen), (4) OncoCarta (Agena Bioscience), and (5) cobas® *KRAS* mutation test (Roche) together with LightMix® kits (TIB MOLBIOL) for the cobas system.

CRC *RAS*Scan™ (SURVEYOR®/WAVE®) uses Transgenomics’ proprietary DNA mismatch-cutting enzyme SURVEYOR® nuclease, with cleavage products separated by denaturing high-pressure liquid chromatography using the WAVE® platform [77]. The nature of the mutation is then confirmed by Sanger sequencing. CRC *RAS*seq™ uses Transgenomics’ proprietary primer sets for PCR amplification and Sanger sequencing of exons 2, 3, and 4 of the *KRAS* and *NRAS* genes [79]. This method was used to identify *RAS* mutation status in mCRC patients included in the PRIME study [14].

Therascreen® *KRAS* and *NRAS* Pyro® kits are based on pyrosequencing technology and consist of two assays: one for codons 12 and 13 and a second for codon 61 [80, 81]. Qiagen has recently launched a new *RAS* Extension Pyro Kit, covering codons 58/59, 117, and 146 of *KRAS* and *NRAS*, which has recently received CE-marked status, although the LOD of this extension kit is not reported [93].

The Agena Bioscience OncoCarta panel includes 12 *KRAS* and eight *NRAS* mutations [82]. Detection is based on the Agena MassARRAY® system, which uses matrix-assisted laser desorption/ionization–time of flight mass spectrometry [94].

The cobas® system works on the basis of high-resolution melting using different PCR reactions together with mutation-specific probes coupled with different fluorochromes using the real-time PCR cobas 480® (Roche) device. After the generation of PCR products, these are melted in a temperature gradient. The melting point of the mutation-specific probe indicates the type of mutation, being specific for either the codon (cobas® *KRAS* mutation test) or the position (LightMix® kits).

The choice of CE-marked *RAS* mutation test by individual laboratories is largely dependent on the available equipment,

Table 4 CE-approved *RAS*-testing kits available in Europe

In vitro diagnostic kit	<i>KRAS</i> ^a	<i>NRAS</i> ^a	Mutant enrichment	Detection method	LOD (%) ^b
CRC <i>RAS</i> Scan™ (SURVEYOR®/WAVE®) [78]	Yes	Yes	None	Mismatched heteroduplex cleavage and DHPLC ^c	2–5
CRC <i>RAS</i> seq™ [79]	Yes	Yes	None	Sanger sequencing	5–10
Therascreen® <i>KRAS</i> and <i>NRAS</i> Pyro [80, 81]	Yes	Yes	None	Pyrosequencing	1–7
Agenda ^d OncoCarta™ [82, 83]	Yes	Yes	None	Mass spectrometry	5–10
LightMix® <i>NRAS</i> ex2–4 <i>KRAS</i> ex4 [90]	Yes	Yes	PCR clamp (LNA)	Melting curve analysis-based	1
Therascreen® <i>KRAS</i> RGQ PCR [84]	Yes	No	Allele-specific probes	RT-PCR	1–6
cobas <i>KRAS</i> mutation test [85]	Yes	No	PCR clamp (TaqMelt™)	Melting curve analysis-based	5
Randox biochip array [86]	Yes	No	None	Array hybridization	1
INFINITI® <i>KRAS</i> [87]	Yes	No	None	Array hybridization	NS
<i>KRAS</i> StripAssay® [88]	Yes	No	PCR clamp (blocker)	Strip hybridization	1
EnteroGen® <i>KRAS</i> [89]	Yes	No	Allele-specific probes	RT-PCR	<1
LightMix® <i>KRAS</i> codon 12/13 [90]	Yes	No	PCR clamp (LNA)	Melting curve analysis-based	1
PNAClamp™ [91]	Yes	No	PCR clamp (PNA)	RT-PCR	<1
RealQuality RI- <i>KRAS</i> MuST [92]	Yes	No	Allele-specific probes	RT-PCR	1

DHPLC denaturing high-pressure liquid chromatography, LNA locked nucleic acid, LOD limit of detection, NS not specified, PCR polymerase chain reaction, PNA peptide nucleic acid, RT-PCR reverse transcriptase polymerase chain reaction

^a Methods may not include all exons or relevant codons

^b Determination methods may vary

^c Confirmation by DNA sequencing recommended

^d Formerly Sequenom

experience, and costs of the test [95]. The rapid change in knowledge of biomarkers and loci implies that development of additional commercial kits is necessary, and the situation for *RAS* screening, with only five available kits covering all loci, highlights the relatively low reactivity of the market.

Laboratory-developed methods

Many laboratory-developed methods for *RAS* testing are used for in vitro diagnostics, and still more methods exist for research-use only [42]; the latter are beyond the scope of this review. This is especially true for NGS, as commercially available kits are almost inaccessible, particularly those of CE-quality. CE-marked assays for NGS could be the next step to ensure the quality of reagents and primer pools. Initiatives to standardize a primer set, such as the community panel from Life Technologies and the Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2, are underway, although such primer sets are currently for research-use only [57]. Most commonly, Sanger sequencing- and pyrosequencing-based laboratory-developed methods are used for *RAS* testing [43]. In some countries, such as France, allele-specific PCR is very popular (Dequeker et al., manuscript submitted). Here, mutant alleles are selectively amplified, making this technique highly sensitive for the detection of mutations in the *RAS* genes. There are many different variations of this general principle (Table 5) [96–100], which is reflected in the many variations of allele-

specific hybridization methods [43]. In general, laboratory-developed methods offer the possibility of more rapid adaptation to new knowledge of biomarkers and loci, although their use requires a high level of experience and quality management within the laboratory. Moreover, mostly as a result of the costs of CE-marked kits, laboratory-developed techniques are very suitable for testing the sensitivity (LOD) and specificity of *RAS* mutation detection, which is important in the maintenance of quality.

Sensitivity of *RAS* testing

The sensitivity of mutation detection is influenced by multiple factors, mostly LOD and the minimum amount of required template. The LOD is dependent on the technique used and might therefore be considered as the technical sensitivity, which is defined by the ratio of the

Table 5 Allele-specific polymerase chain reaction (PCR) methods for *RAS* testing

Amplification refractory mutation system (ARMS) [96]
Restriction endonuclease-mediated selective (REMS) PCR [97]
Fluorescent amplicon generation (FLAG) [98]
Restriction fragment length polymorphisms (RFLP) [99]
Allele-specific ligation detection reaction [100]

measurement signal (mutation) to the background signal (mutant signal in a WT sample) [43]. The sensitivities of available tests range from <1 to 10 % for CE-approved testing methods (Table 4). For laboratory methods, the most sensitive techniques are allele-specific PCR and hybridization assays (0.1–1 %) and gel electrophoresis (e.g., temporal temperature gradient or constant denaturant capillary electrophoresis) (1 %) [43]. Pyrosequencing (5 %), high-resolution melting curve analysis (~10 %), and Sanger sequencing (10–25 %) have a lower level of technical sensitivity [43, 101]. This highlights the benefit of histopathology as the basis of mutation detection: sensitivity can be controlled, as the total amount of tissue—reflecting the amount of template, and relative fraction of neoplastic cells within the tumor (measured as area or total nuclei)—reflecting the LOD, can be determined (semi) quantitatively. For example, a *KRAS* mutation in a small metastatic focus of neoplastic cells in a lymph node can escape detection when entire tissue sections of the lymph node are used for DNA isolation (i.e., normal cell DNA content is far greater than neoplastic cell DNA). After careful microdissection of the neoplastic cells, however, the *KRAS* mutation can unequivocally be identified. Thus, in clinical practice, the sensitivity of the entire process must be considered, not just the sensitivity of the technique being used.

Unfortunately, the sensitivity of mutation detection—which needs to be related to the clinical effect—in the reference studies establishing the validity of *RAS* as a biomarker is unknown. This is because only the technical sensitivity of detection can be derived from the method used and the minimum required neoplastic cell fraction required as input, not the actual values for individual samples. Thus, the clinical significance of high versus lower sensitivity, indicating the number of neoplastic clones with or without a *RAS* mutation, is currently unclear. Achievement of higher sensitivity must, therefore, be weighed against the possibility of false positives or the identification of mutations present only in a small minority of the neoplastic cells, reflecting a small tumor clone population with *RAS* mutations, which is of unknown clinical relevance. Additionally, sensitivity depends on the experience of the laboratory, as results for the LOD of Sanger sequencing were found to be similar to allele-specific methods during blind testing [102]. Furthermore, a recent publication suggests that tumors containing <1 % mutant *KRAS* DNA allele-fraction in microdissected samples might still benefit from treatment with anti-EGFR agents [103]. Overall, it is important to take into account histopathological information from the tissue samples used for mutation detection in future clinical studies, to better define cutoffs for sensitivity and clinical response, and to improve the benefit to patients of targeted therapies.

Timing of testing

Turnaround times for testing remain a key challenge for pathologists and oncologists, and it is important that a strategy is in place to optimize the process, whether this is through stratified reflex testing based on patient risk factors or through logistical management of new or archived tumor samples. In general, European EQA schemes recommend that the process of obtaining a *RAS* test result should take no more than 10 working days [104], and it is important that pathology and oncology centers work together to address barriers that prevent prompt turnaround of test requests. NGS processes generally take more than 2 days to complete, which is the main bottleneck when choosing new technology in comparison to conventional methods.

Reflex sampling involves the immediate sampling and testing of mCRC at the time of diagnostic biopsy. The major benefit of reflex testing is that information on *RAS* status is available as soon as EGFR-targeted therapies are considered, so no time is lost in starting treatment. Reflex testing in which all colorectal cancers are tested to anticipate the risk of metastasis is also possible, although this remains an area of debate. Underlying *RAS* mutations appear to occur early in tumor development, and therefore, testing for *RAS* mutations is potentially informative, although the prognostic significance of such information is not known. While delays in obtaining *RAS* test results can affect treatment strategies, the implementation of reflex testing is likely to be associated with a high degree of unnecessary testing. Whatever the timing of testing, it is important that interpretation of results must take into account the patient's clinical history.

Bioinformatics

The function of the bioinformatics pipeline with regard to *RAS* analysis is to convert the raw sequencing data into clinically useful information through the use of appropriate software algorithms [105]. Integration of NGS technologies into the clinical setting has the potential to verify suggestive relationships and remove ambiguity more quickly than current approaches. Validation of the output from such algorithms can be achieved through the use of reference sequences, control samples containing specific *RAS* variants, and by comparing the output with that from alternative sequencing platforms. Guidelines issued by the National Collaborative Study of Dutch Genome Diagnostic Laboratories state that data analysis and quality checks can be divided into five main steps: base calling and quality assessment of raw data, mapping of reads against a given reference sequence, enrichment analysis, coverage analysis, and variant calling and annotation [106]. The guidelines further state that minimal quality criteria must be established in each laboratory to determine the overall quality

of platform performance, although the specific criteria usually depend on the sequencing platform being used.

The initial *RAS* sequencing report should include all variants that were detected against a stated reference sequence, regardless of their clinical significance, although this information should be used to annotate each variant as far as possible (including whether or not clinical validation has been reported [105]. The report should also include information on the test used, including any important limitations, along with base quality scores and confirmatory testing. Notably, a study of genome sequencing across 30 international groups showed that, while there was generally good consistency across bioinformatics analysis and interpretation, there was a lack of concordance with regard to interpretation, report content, and patient consent procedures [107]. Suggested reasons for differences between groups included limited quality control measures; different read aligners and variant calling pipelines; variations in performance of analysis tools, software, and filters; errors in programming or manual analysis; and poor-quality data [105, 107].

The role and importance of quality assurance programs

Given the large variety of techniques and the importance of laboratories' experience, it is essential to test and validate centers' performance with standardized EQA schemes. Use of CE-marked kits or fully validated methods involves proof that quality is maintained after the implementation of the marker in the laboratory. Nevertheless, EQA validation should be done periodically to obtain assurance about the laboratory's quality.

Guideline recommendations for a European EQA program to ensure accuracy and proficiency in *KRAS* mutation testing were published in 2008 [42]. A number of EQA programs are now available (Table 6), [42, 108–114] such as the European program established by the European Society of Pathology (ESP) for testing biomarker mutations in colorectal cancer [42] or the German Quality Initiative in Pathology (QuIP)

(Jung et al., manuscript in preparation). The European program aims to ensure optimal accuracy and proficiency in biomarker testing in colorectal cancer across all participating countries [115] and is organized in collaboration with a European working group and the Biomedical Quality Assurance Research Unit of the University of Leuven [115]. The program provides recommendations and overviews of laboratory methods, standardized operating procedures, and accreditation criteria relevant for *RAS* mutation testing [42, 115, 116].

The Organization for Economic Co-operation and Development (OECD) guidelines for Quality Assurance in Molecular Genetic Testing address processes relating to genetic testing for variations in germline DNA sequences that are also applicable to somatic DNA testing. The guidelines cover general principles and best practices, EQA systems, proficiency testing (monitoring the quality of laboratory performance), quality of result reporting, and education and training standards for laboratory personnel [117].

In March 2012, medical oncologists, pathologists, geneticists, molecular biologists, EQA providers and representatives from pharmaceutical industries developed a guideline to harmonize the standards applied by EQA schemes in molecular pathology. The guideline comprises recommendations on the organization of an EQA scheme, defining the criteria for reference laboratories, requirements for EQA test samples, and the number of samples that are needed for an EQA scheme [118]. All EQA schemes should be developed by an expert group with a representing EQA provider, which is also responsible for all organizational aspects and operation of the scheme according to International Organization for Standardization (ISO) 17043 standards. For each scheme, a team coordinator is responsible for the selection, distribution and receipt of cases, and the analysis and reporting of results. Importantly, the samples used in an EQA scheme should reflect the diagnostic and clinical reality as closely as possible, and the turnaround time, as defined by the EQA provider, should reflect the common clinical situation. For a reliable evaluation, at least 10 samples per laboratory need to be analyzed per year and can be provided in a single batch, or in multiple smaller batches. The limit for poor performance should be defined as a score 18 out of 20, with no false-positive or false-negative results. Finally, EQA providers should be encouraged to make the general reports available in the public domain. The ESP Colon EQA scheme is organized in accordance with these recommendations and with the OECD best practice guidelines [42]. OECD Principles of Good Laboratory Practice state that individual testing facilities should have a documented QA program to ensure that studies performed are in compliance with principles of good laboratory practice [119]. A number of authors have stated that participation in internal QA schemes, as well as external schemes, should be mandatory for diagnostic pathology laboratories

Table 6 Quality assurance programs for *RAS* testing in colorectal cancer

AIOM-SIAPEC Italian Program for EQA in molecular pathology [112]
College of American Pathologists [108]
European Molecular Genetics Quality Network [110]
European Society for Pathology [42]
Gen&Tiss French Program–French Cancer Institute (INCa)–AFAQAP–GFCO [113, 114]
German Society for Clinical Chemistry and Laboratory Medicine [111]
German Society for Pathology–QuIP (Quality Initiative Pathology) ^a
United Kingdom National External Quality Assessment Service [109]

^a Jung et al. manuscript in preparation

[95, 120]. Furthermore, the validation or verification of testing methods is a formal requirement for the accreditation of laboratories according to the major international standard applicable to medical testing laboratories: ISO 15189 [121, 122].

The need for QA schemes was highlighted by recent research carried out under the European QA program [45], in which 27 % of participating laboratories genotyped at least 10 % of samples incorrectly, although <5 % of distributed specimens overall were genotyped incorrectly. Errors included false negatives, false positives, and incorrectly genotyped mutations. In addition, 20 % of laboratories reported a technical error for at least one sample. It is encouraging, however, that the majority of laboratories made no mistakes. There are now sufficient quality-assured laboratories to allow *RAS* testing across Europe, and even a small number of EQA rounds can improve quality to a high level [123].

In the future, as new techniques become routinely available, existing EQA programs will need to evolve to address the issues arising from the use of NGS and other increasingly sensitive techniques.

Conclusions

Pathologists have a key role to play in ensuring that patients receive optimal and appropriate treatment for mCRC. Up-front tumor *RAS* testing—rather than solely *KRAS* exon 2 testing—is critical for patients with mCRC, as *RAS* mutations predict a lack of response to anti-EGFR therapy [14]. The more extensive use of biomarkers is already included in relevant treatment guidelines from ESMO, the NCCN, and the AIO [38, 39, 41] and is increasingly recognized as an essential component of mCRC diagnosis and work-up. The reference methods are sequencing: initially the Sanger method and subsequently pyrosequencing. More recently, NGS techniques for mutational analysis have been developed, the key methods applied to molecular pathology being parallel semiconductor and reversible termination sequencing. Some CE-approved kits are commercially available, but most apply only to *KRAS*. Only five kits currently cover both *KRAS* and *NRAS* genes, although even these do not yet include all potentially relevant mutations. Further kit development should be more flexible to allow for evolution of new biomarkers. In addition, many laboratories have their own in-house methods for assessing mutational status and have the ability to more rapidly adapt testing to clinically validated biomarkers. The different analysis techniques have differing technical sensitivities for detecting mutations, although the ideal process sensitivity threshold—including that relating to the neoplastic cell burden in the sample—and the value of tests with a very high sensitivity are currently unknown. More comprehensive planning of clinical studies in the future is therefore required.

Appropriate tumor sampling and prompt delivery of material to diagnostic laboratories is critical for accurate and timely *RAS* testing. Processes to ensure that the turnaround time from sample request to *RAS* evaluation is as efficient as possible need to be implemented on a national and center-specific level to ensure that patients have the option to receive the most appropriate treatment.

EQA programs play an important role in ensuring that *RAS* testing is carried out to a high standard, especially when laboratory-developed methods are used. EQA testing should include “difficult” samples that are at the LOD of the assay being used and should also test the entire chain of analysis, as there are many different steps involved, from preparation of the FFPE block and DNA to the actual molecular testing, results, and bioinformatic reports.

Finally, *RAS* testing is still a moving field, and important future directions include the extension of NGS to an increased number of biomarkers and the extension of quantitative methods to include circulating plasma DNA.

Acknowledgments Medical writing assistance with this manuscript was provided by Daniel Booth, PhD (Bioscript Medical, UK) and funded by Amgen (Europe) GmbH.

Authors' contributions All authors were involved in critically reviewing the manuscript at every stage of development and in the decision to submit the manuscript for publication.

Compliance with ethical standards

Conflict of interest JHJM Van Krieken has participated in advisory boards for Amgen, Merck Serono, GlaxoSmithKline, and Sakura and has received honoraria and research grants from these companies. N Normanno has participated in advisory boards for Amgen, AstraZeneca, Merck Serono, and Roche. MJL Ligtenberg has received research funding from Amgen and AstraZeneca and has participated in advisory boards for AstraZeneca. E Rouleau has received research funding from Amgen and Roche and honoraria from AstraZeneca and GlaxoSmithKline. SD Patterson is a stockholder and former employee of Amgen Inc. A Jung has participated in advisory boards and received honoraria from Amgen, AstraZeneca, Boehringer, Celgene, GlaxoSmithKline, Merck Serono, Pfizer, Qiagen, and Roche.

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References

1. Heinemann V, Douillard JY, Ducreux M, Peeters M (2013) Targeted therapy in metastatic colorectal cancer—an example of personalised medicine in action. *Cancer Treat Rev* 39:592–601
2. Vijayaraghavan A, Efrusy MB, Goke B, Kirchner T, Santas CC, Goldberg RM (2012) Cost-effectiveness of *KRAS* testing in

- metastatic colorectal cancer patients in the United States and Germany. *Int J Cancer* 131:438–445
3. Biomarkers Working Group (2001) Biomarkers and surrogate end-points: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 69:89–95
 4. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F (2013) Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 49:1374–1403
 5. Siddiqui AD, Piperdi B (2010) *KRAS* mutation in colon cancer: a marker of resistance to EGFR-I therapy. *Ann Surg Oncol* 17:1168–1176
 6. Lièvre A, Artru P, Guiu M, Laurent-Puig P, Merlin JL, Sabourin JC, Viguier J, Bastie A, Seronde A, Ducreux M (2013) The *KRAS* mutation detection within the initial management of patients with metastatic colorectal cancer: a status report in France in 2011. *Eur J Cancer* 49:2126–2133
 7. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD (2008) Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 26:1626–1634
 8. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jasssem J, Rivera F, Kocakova I, Ruff P, Blasinska-Morawiec M, Smakal M, et al. (2010) Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 28:4697–4705
 9. Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, Andre T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, et al. (2010) Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol* 28:4706–4713
 10. Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, et al. (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 360:1408–1417
 11. Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zube A, Celik I, Rougier P, Ciardiello F (2011) Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor *KRAS* and *BRAF* mutation status. *J Clin Oncol* 29:2011–2019
 12. Peeters M, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, Andre T, Chan E, Lordick F, Punt CJA, Strickland A, Wilson G, Ciuleanu T-E, Roman L, et al. (2014) Analysis of *KRAS*/*NRAS* mutations in phase 3 study 20050181 of panitumumab (pmab) plus FOLFIRI versus FOLFIRI for second-line treatment (tx) of metastatic colorectal cancer (mCRC). *J Clin Oncol* 32 (Suppl 3):Abstract #LBA387
 13. Tejpar S, Peeters M, Gelderblom H, Humblet Y, Vermoken J, De Hertogh G, Nippgen J, von Heydebreck A, Stroh C, Van Cutsem E (2008) Relationship of efficacy with *KRAS* status (wild type [wt] vs mutant [mt]) in patients with irinotecan-refractory metastatic colorectal cancer (mCRC), treated with irinotecan and escalating doses of cetuximab: preliminary data from the EVEREST study. *Ann Oncol* 19 (Suppl 8):viii125 (Abstract #358P)
 14. Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jasssem J, Rivera F, Kocakova I, Ruff P, Blasinska-Morawiec M, Smakal M, et al. (2013) Panitumumab-FOLFOX4 treatment and *RAS* mutations in colorectal cancer. *N Engl J Med* 369:1023–1034
 15. Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, Loos AH, Zube A, Koralewski P (2009) Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 27:663–671
 16. Pritchard CC, Grady WM (2011) Colorectal cancer molecular biology moves into clinical practice. *Gut* 60:116–129
 17. Heinemann V, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Hielscher J, Scholz M, et al. (2014) FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol* 15:1065–1075
 18. Schwartzberg LS, Rivera F, Karthaus M, Fasola G, Canon JL, Hecht JR, Yu H, Oliner KS, Go WY (2014) PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type *KRAS* exon 2 metastatic colorectal cancer. *J Clin Oncol* 32:2240–2247
 19. Peeters M, Oliner KS, Parker A, Siena S, Van Cutsem E, Huang J, Humblet Y, Van Laethem JL, Andre T, Wizeorek J, Reese D, Patterson SD (2013) Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. *Clin Cancer Res* 19:1902–1912
 20. Peeters M, Douillard JY, Van Cutsem E, Siena S, Zhang K, Williams R, Wizeorek J (2013) Mutant *KRAS* codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. *J Clin Oncol* 31:759–765
 21. De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, Lamba S, Arena S, Frattini M, Piessevaux H, Van Cutsem E, O'Callaghan CJ, Khambata-Ford S, Zalberg JR, Simes J, et al. (2010) Association of *KRAS* p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 304:1812–1820
 22. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, et al. (2010) Effects of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 11:753–762
 23. Andre T, Blons H, Mabro M, Chibaudel B, Bachet JB, Tournigand C, Bannamoun M, Artru P, Nguyen S, Ebenezzer C, Aissat N, Cayre A, Penault-Llorca F, Laurent-Puig P, de Gramont A, GERCOR (2013) Panitumumab combined with irinotecan for patients with *KRAS* wild-type metastatic colorectal cancer refractory to standard chemotherapy: a GERCOR efficacy, tolerance, and translational molecular study. *Ann Oncol* 24:412–419
 24. Loupakis F, Ruzzo A, Cremolini C, Vincenzi B, Salvatore L, Santini D, Masi G, Stasi I, Canestrari E, Rulli E, Floriani I, Bencardino K, Galluccio N, Catalano V, Tonini G, et al. (2009) *KRAS* codon 61, 146 and *BRAF* mutations predict resistance to cetuximab plus irinotecan in *KRAS* codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer* 101:715–721
 25. Peeters M, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, Andre T, Chan E, Lordick F, Punt CJA, Strickland A, Wilson G, Ciuleanu TE, Roman L, et al. (2014) Updated analysis of *KRAS*/*NRAS* and *BRAF* mutations in study 20050181 of panitumumab (pmab) plus FOLFIRI for second-line treatment (tx) of metastatic colorectal cancer (mCRC). Poster presented at ASCO 2014
 26. Patterson SD, Peeters M, Siena S, Van Cutsem E, Humblet Y, Van Laethem JL, Andre T, Tian Y, Sidhu R, Oliner KS (2013) Comprehensive analysis of *KRAS* and *NRAS* mutations as

- predictive biomarkers for single agent panitumumab (pmab) response in a randomized, phase III metastatic colorectal cancer (mCRC) study (20020408). *J Clin Oncol* 31:3617(Abtract)
27. Seymour MT, Brown SR, Middleton G, Maughan T, Richman S, Gwyther S, Lowe C, Seligmann JF, Wadsley J, Maisey N, Chau I, Hill M, Dawson L, Falk S, O'Callaghan A, et al. (2013) Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. *Lancet Oncol* 14:749–759
 28. Van Cutsem E, Lenz HJ, Köhne CH, Heinemann V, Tejpar S, Melezinek I, Beier F, Stroh C, Rougier P, van Krieken JH, Ciardiello F (2015) Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J Clin Oncol* 33:692–700
 29. Maughan TS, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, Idziaszczyk S, Harris R, Fisher D, Kenny SL, Kay E, Mitchell JK, Madi A, Jasani B, James MD, et al. (2011) Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 377:2103–2114
 30. Stintzing S, Jung A, Rossius L, Modest DP, Fischer von Weikersthal L, Decker T, Kiani A, Al-Batran S-E, Vehling-Kaiser U, Heintges T, Moehler M, Scheithauer W, Kirchner T, Heinemann V (2014) Mutations within the EGFR signaling pathway: influence on efficacy in FIRE-3—a randomized phase III study of FOLFIRI plus cetuximab or bevacizumab as first-line treatment for wild-type (WT) KRAS (exon 2) metastatic colorectal cancer (mCRC) patients. *J Clin Oncol* 32 (Suppl 3):Abstract 445. Poster presented at ASCO 2014
 31. Bokemeyer C, Kohne C-H, Ciardiello F, Lenz H-J, Heinemann V, Klinkhardt U, Beier F, Duecker K, Tejpar S (2014) Treatment outcome according to tumor RAS mutation status in OPUS study patients with metastatic colorectal cancer (mCRC) randomized to FOLFOX4 with/without cetuximab. *J Clin Oncol* 32 (Suppl 5s): Abstract 3505. Poster presented at ASCO 2014
 32. Peeters M, Kafatos G, Taylor A, Gastanaga V, Yu H, Oliner KS, Hechmati G, Terwey J-H, van Krieken JH (2015) Prevalence of RAS mutations among patients with metastatic colorectal cancer by country and region in randomized clinical trials: a pooled analysis. Presented at ASCO-GI, San Francisco, CA, USA
 33. Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, Karapetis CS (2015) Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann Oncol* 26: 13–21
 34. Amgen Europe BV (2013) Vectibix. EPAR product information. Amgen Europe B.V., Breda
 35. Merck KGaA (2009) Erbitux. EPAR product information. Merck KGaA, Darmstadt
 36. Finkelstein SD, Sayegh R, Christensen S, Swalsky PA (1993) Genotypic classification of colorectal adenocarcinoma. Biologic behavior correlates with K-ras-2 mutation type. *Cancer* 71:3827–3838
 37. Finkelstein SD, Sayegh R, Bakker A, Swalsky P (1993) Determination of tumor aggressiveness in colorectal cancer by K-ras-2 analysis. *Arch Surg* 128:526–531 discussion 531–522
 38. National Comprehensive Cancer Network (2014) NCCN Clinical Practice Guidelines in Oncology™. Colon cancer. V3. National Comprehensive Cancer Network, Jamestown, PA
 39. AIO (2013) Statement der AIO-KRK Leitgruppe für die Antikörpertherapie in Kombination mit einer Chemotherapie. Arbeitsgemeinschaft Internistische Onkologie in der Deutschen Krebsgesellschaft e.V., Berlin
 40. Landelijke Werkgroep Gastro Intestinale Tumoren (2014) Algemeen. <http://www.oncolinenl/colorectaalcarcinoom>. Accessed December 2014
 41. Van Cutsem E, Cervantes A, Nordlinger B, Arnold D (2014) Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 25(Suppl 3): iii1–iii9
 42. van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, Quirke P, Flejou JF, Plato HT, De Hertogh G, Jares P, Langner C, Hoefler G, Ligtenberg M, Tiniakos D, et al. (2008) KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch* 453:417–431
 43. Shackelford RE, Whitting NA, McNab P, Japa S, Coppola D (2012) KRAS testing: a tool for the implementation of personalized medicine. *Genes Cancer* 3:459–466
 44. Monzon FA, Ogino S, Hammond ME, Halling KC, Bloom KJ, Nikiforova MN (2009) The role of KRAS mutation testing in the management of patients with metastatic colorectal cancer. *Arch Pathol Lab Med* 133:1600–1606
 45. Tembuysers L, Ligtenberg MJ, Normanno N, Delen S, van Krieken JH, Dequeker EM (2014) Higher quality of molecular testing, an unfulfilled priority: results from external quality assessment for KRAS mutation testing in colorectal cancer. *J Mol Diagn* 16:371–377
 46. Morey M, Fernandez-Marmiesse A, Castineiras D, Fraga JM, Couce ML, Cocho JA (2013) A glimpse into past, present, and future DNA sequencing. *Mol Genet Metab* 110:3–24
 47. Tol J, Dijkstra JR, Vink-Borger ME, Nagtegaal ID, Punt CJ, van Krieken JH, Ligtenberg MJ (2010) High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. *J Cell Mol Med* 14:2122–2131
 48. Carotenuto P, Roma C, Rachiglio AM, Tatangelo F, Pinto C, Ciardiello F, Nappi O, Iaffaioli RV, Botti G, Normanno N (2010) Detection of KRAS mutations in colorectal carcinoma patients with an integrated PCR/sequencing and real-time PCR approach. *Pharmacogenomics* 11:1169–1179
 49. Milbury CA, Li J, Makrigiorgos GM (2009) PCR-based methods for the enrichment of minority alleles and mutations. *Clin Chem* 55: 632–640
 50. Carotenuto P, Roma C, Cozzolino S, Fenizia F, Rachiglio AM, Tatangelo F, Iannaccone A, Baron L, Botti G, Normanno N (2012) Detection of KRAS mutations in colorectal cancer with Fast COLD-PCR. *Int J Oncol* 40:378–384
 51. Parsons BL, Heflich RH (1997) Genotypic selection methods for the direct analysis of point mutations. *Mutat Res* 387:97–121
 52. Gocke CD, Benko FA, Kopreski MS, Evans DB (2000) Enrichment methods for mutation detection. *Ann N Y Acad Sci* 906:31–38
 53. Chang YS, Er TK, Lu HC, Yeh KT, Chang JG (2014) Detection of KRAS codon 12 and 13 mutations by mutant-enriched PCR assay. *Clin Chim Acta* 436C:169–175
 54. Hoff KJ (2009) The effect of sequencing errors on metagenomic gene prediction. *BMC Genomics* 10:520
 55. Ibrahim S, Seth R, O'Sullivan B, Fadhill W, Taniere P, Ilyas M (2010) Comparative analysis of pyrosequencing and QMC-PCR in conjunction with high resolution melting for KRAS/BRAF mutation detection. *Int J Exp Pathol* 91:500–505
 56. Balzer S, Malde K, Jonassen I (2011) Systematic exploration of error sources in pyrosequencing flowgram data. *Bioinformatics* 27:i304–i309
 57. Ciardiello F, Normanno N, Maiello E, Martinelli E, Troiani T, Piscconti S, Giuliani F, Barone C, Carteni G, Rachiglio AM, Montesarchio V, Tonini G, Rizzi D, Cinieri S, Bordonaro R, et al. (2014) Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next-generation sequencing: findings from the CAPRI-GOIM trial. *Ann Oncol* 25:1756–1761

58. Moorthie S, Mattocks CJ, Wright CF (2011) Review of massively parallel DNA sequencing technologies. *HUGO J* 5:1–12
59. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, Hall KP, Evers DJ, Barnes CL, Bignell HR, Boutell JM, Bryant J, Carter RJ, Keira CR, Cox AJ, et al. (2008) Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* 456:53–59
60. Roche (2014) The technology. <http://454.com/products/technology.asp>. Accessed December 2014
61. Illumina (2014) Sequencing systems. <http://www.illumina.com/systems/sequencingilmn>. Accessed December 2014
62. Life Technologies (2011) Ion Torrent amplicon sequencing. http://www.3appliedbiosystems.com/cms/groups/applied_markets_marketing/documents/generaldocuments/cms_094273.pdf. Accessed December 2014
63. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 13: 341
64. Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M (2012) Comparison of next-generation sequencing systems. *J Biomed Biotechnol* 2012:251364
65. Ross MG, Russ C, Costello M, Hollinger A, Lennon NJ, Hegarty R, Nusbaum C, Jaffe DB (2013) Characterizing and measuring bias in sequence data. *Genome Biol* 14:R51
66. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, Leamon JH, Johnson K, Milgrew MJ, Edwards M, Hoon J, Simons JF, Marran D, Myers JW, Davidson JF, et al. (2011) An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 475:348–352
67. Tops BB, Normanno N, Kurth H, Amato E, Mafficini A, Rieber N, Le Corre D, Rachiglio AM, Reiman A, Sheils O, Noppen C, Lacroix L, Cree IA, Scarpa A, Ligtenberg MJ, Laurent-Puig P (2015) Development of a semi-conductor sequencing-based panel for genotyping of colon and lung cancer by the OncoNetwork Consortium. *BMC Cancer* 15:26
68. Vogelstein B, Kinzler KW (1999) Digital PCR. *Proc Natl Acad Sci U S A* 96:9236–9241
69. Li M, Chen WD, Papadopoulos N, Goodman SN, Bjerregaard NC, Laurberg S, Levin B, Juhl H, Arber N, Moinova H, Durkee K, Schmidt K, He Y, Diehl F, Velculescu VE, et al. (2009) Sensitive digital quantification of DNA methylation in clinical samples. *Nat Biotechnol* 27:858–863
70. Hiatt JB, Pritchard CC, Salipante SJ, O’Roak BJ, Shendure J (2013) Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. *Genome Res* 23: 843–854
71. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, et al. (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 6:224ra224
72. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B (2003) Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc Natl Acad Sci U S A* 100:8817–8822
73. Tejpar S, Lenz H-J, Köhne C-H, Heinemann V, Ciardiello F, Esser R, Beier F, Stroh C, Duecker C, Bokemeyer C (2014) Effect of KRAS and NRAS mutations on treatment outcomes in patients with metastatic colorectal cancer (mCRC) treated first-line with cetuximab plus FOLFIRI: new results from the OPUS study. *J Clin Oncol* 32 (Suppl 3):Abstract #LBA444
74. Ciardiello F, Lenz H-J, Köhne CH, Heinemann V, Tejpar S, Esser R, Beier F, Stroh C, Duecker C, Van Cutsem E (2014) Effect of KRAS and NRAS mutational status on first-line treatment with FOLFIRI plus cetuximab in patients with metastatic colorectal cancer (mCRC): new results from the CRYSTAL trial. *J Clin Oncol* 32 (Suppl 3):Abstract #LBA443
75. Azuara D, Ginesta MM, Gausachs M, Rodriguez-Moranta F, Fabregat J, Busquets J, Pelaez N, Boadas J, Galter S, Moreno V, Costa J, de Oca J, Capella G (2012) Nanofluidic digital PCR for KRAS mutation detection and quantification in gastrointestinal cancer. *Clin Chem* 58:1332–1341
76. Taly V, Pekin D, Benhaim L, Kotsopoulos SK, Le CD, Li X, Atochin I, Link DR, Griffiths AD, Pallier K, Blons H, Bouche O, Landi B, Hutchison JB, Laurent-Puig P (2013) Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. *Clin Chem* 59: 1722–1731
77. European Commission (1998) Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices. http://www.emergogroup.com/sites/default/files/file/in-vitro-diagnostics-directive-98-79-ec-v2_0.pdf. Accessed December 2014
78. Transgenomic Inc. (2013) Instructions for use for the CRC RAScan™ Combination Kit. <http://worldtransgenomic.com/files/literature/482427-EN.pdf>. Accessed December 2014
79. Transgenomic Inc. (2013) Instructions for use for the CRC RASseq™ kit. <http://worldtransgenomic.com/files/literature/482482-EN.pdf>. Accessed December 2014
80. Qiagen (2011) Therascreen KRAS Pyro Kit handbook v1. <http://tinyurl.com/lkbdk2f>. Accessed December 2014
81. Qiagen (2011) Therascreen NRAS Pyro Kit handbook v1. <http://tinyurl.com/m64zerb>. Accessed December 2014
82. Agena Bioscience (2014) OncoCarta™ panel v1.0. http://agenabio.com/wp-content/uploads/2015/05/51-20010R2.0-OncoCarta-Panel-v1.0-Flyer_WEB.pdf. Accessed July 2015
83. Sequenom (2010) A rapid and highly sensitive method for somatic mutation profiling using the Sequenom MassARRAY® system. <http://www.biotechniques.com/protocols/A-rapid-and-highly-sensitive-method-for-somatic-mutation-profiling—using-the-Sequenom-MassARRAY-System/biotechniques-312575.html>. Accessed December 2014
84. Qiagen (2012) Therascreen KRAS RGQ PCR kit handbook. http://www.accessdatafda.gov/cdrh_docs/pdf11/P110030c.pdf. Accessed December 2014
85. Roche (2014) cobas® KRAS mutation test. <http://molecularroche.com/assays/Pages/cobasKRASMutationTest.aspx>. Accessed December 2014
86. Randox (2014) KRAS/BRAF/PIK3CA array. <http://www.randox.com/kras-colorectal-molecular-test.php>. Accessed December 2014
87. AutoGenomics (2014) INFINITI® KRAS. <http://www.autogenomics.com/?q=node/199>. Accessed December 2014
88. Farina Sarasqueta A, Moerland E, de Bruyne H, de Graaf H, Vrancken T, van Lijnschoten G, van den Brule AJ (2011) SNaPshot and StripAssay as valuable alternatives to direct sequencing for KRAS mutation detection in colon cancer routine diagnostics. *J Mol Diagn* 13:199–205
89. EnteroGen (2014) K-Ras and B-Raf mutation analysis kits. <http://www.entrogen.com/web2/k-ras-braf-mutation-test-kit>. Accessed December 2014
90. Tib Molbiol (2014) Oligonucleotides, design for PCR and LightCycler. http://www.roche-ases/logs/LightMix%C2%AE_40-0654-16_NRAS-KRAS_V131201a.pdf. Accessed December 2014
91. Panagene (2012) PNAclamp™ KRAS mutation detection kit (Ver. 2). http://www.pnabio.com/pdf/PNAclamp_KRAS_Manual_v12.pdf. Accessed December 2014
92. AB Analitica (2014) REALQUALITY RI-KRAS MuST technical leaflet. <http://www.abanalitica.it/download/?ID=545>. Accessed December 2014

93. Qiagen (2013) RAS extension pyro kit handbook. <http://tinyurl.com/qedz9wm>. Accessed December 2014
94. Oeth P, del Mistro G, Marmellos G, Shi T, van den Boom D (2009) Qualitative and quantitative genotyping using single base primer extension coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MassARRAY). *Methods Mol Biol* 578:307–343
95. Domagala P, Hybiak J, Sulzyc-Bielicka V, Cybulski C, Rys J, Domagala W (2012) KRAS mutation testing in colorectal cancer as an example of the pathologist's role in personalized targeted therapy: a practical approach. *Pol J Pathol* 63:145–164
96. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* 17:2503–2516
97. Mixich F, Ioana M, Voinea F, Saftoiu A, Ciurea T (2007) Noninvasive detection through REMS-PCR technique of K-ras mutations in stool DNA of patients with colorectal cancer. *J Gastrointest Liver Dis* 16:5–10
98. Amicarelli G, Shehi E, Makrigiorgos GM, Adlerstein D (2007) FLAG assay as a novel method for real-time signal generation during PCR: application to detection and genotyping of KRAS codon 12 mutations. *Nucleic Acids Res* 35:e131
99. Kimura K, Nagasaka T, Hoshizima N, Sasamoto H, Notohara K, Takeda M, Kominami K, Iishii T, Tanaka N, Matsubara N (2007) No duplicate KRAS mutation is identified on the same allele in gastric or colorectal cancer cells with multiple KRAS mutations. *J Int Med Res* 35:450–457
100. Khanna M, Park P, Zirvi M, Cao W, Picon A, Day J, Paty P, Barany F (1999) Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors. *Oncogene* 18:27–38
101. Tsiatis AC, Norris-Kirby A, Rich RG, Hafez MJ, Gocke CD, Eshleman JR, Murphy KM (2010) Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. *J Mol Diagn* 12:425–432
102. Blons H, Rouleau E, Charrier N, Chatellier G, Cote JF, Pages JC, de Fraipont F, Boyer JC, Merlio JP, Morel A, Gorisse MC, de Cremoux P, Leroy K, Milano G, Ouafik L, et al. (2013) Performance and cost efficiency of KRAS mutation testing for metastatic colorectal cancer in routine diagnosis: the MOKAECM study, a nationwide experience. *PLoS One* 8:e68945
103. Laurent-Puig P, Pekin D, Normand C, Kotsopoulos SK, Nizard P, Perez-Toralla K, Rowell R, Olson J, Srinivasan P, Le Corre D, Hor T, El Harrak Z, Li X, Link DR, Bouche O, et al. (2015) Clinical relevance of KRAS-mutated subclones detected with picodroplet digital PCR in advanced colorectal cancer treated with anti-EGFR therapy. *Clin Cancer Res* 21:1087–1097
104. van Krieken JH, Siebers AG, Normanno N (2013) European consensus conference for external quality assessment in molecular pathology. *Ann Oncol* 24:1958–1963
105. Bennett NC, Farah CS (2014) Next-generation sequencing in clinical oncology: next steps towards clinical validation. *Cancers (Basel)* 6:2296–2312
106. Weiss MM, Van der Zwaag B, Jongbloed JD, Vogel MJ, Brüggewirth HT, Lekanke Deprez RH, Mook O, Ruivenkamp CA, van Slegtenhorst MA, van den Wijngaard A, Waisfisz Q, Nelen MR, van der Stoep N (2013) Best practice guidelines for the use of next-generation sequencing applications in genome diagnostics: a national collaborative study of Dutch genome diagnostic laboratories. *Hum Mutat* 34:1313–1321
107. Brownstein CA, Beggs AH, Homer N, Merriman B, Yu TW, Flannery KC, DeChene ET, Towne MC, Savage SK, Price EN, Holm IA, Luquette LJ, Lyon E, Majzoub J, Neupert P, et al. (2014) An international effort towards developing standards for best practices in analysis, interpretation and reporting of clinical genome sequencing results in the CLARITY Challenge. *Genome Biol* 15: R53
108. College of American Pathologists (2014) Accreditation and laboratory improvement. http://www.cap.org/apps/capportal?_nfpb=true&_pageLabel=accreditation. Accessed December 2014
109. UK NEQAS (2014) United Kingdom National External Quality Assessment Service. <http://www.ukneqas.org.uk/content/PageServerasp?S=977247289&C=1252&CID=1&type=G>. Accessed December 2014
110. EMQN (2014) European Molecular Genetics Quality Network. <http://www.emqn.org>. Accessed December 2014
111. DGKL (2014) The German Society For Clinical Chemistry And Laboratory Medicine. <http://www.dgkl.de/>. Accessed December 2014
112. Normanno N, Pinto C, Castiglione F, Bardelli A, Gambacorta M, Botti G, Nappi O, Siena S, Ciardiello F, Taddei G, Marchetti A (2011) KRAS mutations testing in colorectal carcinoma patients in Italy: from guidelines to external quality assessment. *PLoS One* 6: e29146
113. European Society of Pathology (2013) Gen&Tiss. http://kraseqascheme.org/info/public/gen_tissx.html. Accessed December 2014
114. Gen&Tiss (2014) Accueil. <http://www.genetiss.org/>. Accessed December 2014
115. European Society of Pathology (2014) Colon external quality assessment scheme. <http://kraseqascheme.org/>. Accessed December 2014
116. Cree IA, Deans Z, Ligtenberg MJ, Normanno N, Edsjo A, Rouleau E, Sole F, Thunnissen E, Timens W, Schuurin E, Dequeker E, Murray S, Dietel M, Groenen P, van Krieken JH (2014) Guidance for laboratories performing molecular pathology for cancer patients. *J Clin Pathol* 67:923–931
117. OECD (2014) Guidelines for quality assurance in genetic testing. <http://www.oecd.org/sti/biotech/oecdguidelinesforqualityassuranceingeneticstesting.htm>. Accessed December 2014
118. van Krieken JH, Normanno N, Blackhall F, Boone E, Botti G, Carneiro F, Celik I, Ciardiello F, Cree IA, Deans ZC, Edsjo A, Groenen PJ, Kamarainen O, Kreipe HH, Ligtenberg MJ, et al. (2013) Guideline on the requirements of external quality assessment programs in molecular pathology. *Virchows Arch* 462:27–37
119. OECD (1998) Principles on good laboratory practice. <http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=en/vmc/chem%2898%2917&doclanguage=en>. Accessed December 2014
120. Dubbink HJ, Deans ZC, Tops BB, van Kemenade FJ, Koljenovic S, van Krieken HJ, Blokx WA, Dinjens WN, Groenen PJ (2014) Next generation diagnostic molecular pathology: critical appraisal of quality assurance in Europe. *Mol Oncol* 8:830–839
121. Berwouts S, Morris MA, Dequeker E (2010) Approaches to quality management and accreditation in a genetic testing laboratory. *Eur J Hum Genet* 18(Suppl 1):S1–19
122. Mattocks CJ, Morris MA, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, Muller CR, Pratt V, Wallace A (2010) A standardized framework for the validation and verification of clinical molecular genetic tests. *Eur J Hum Genet* 18:1276–1288
123. Deans ZC, Bilbe N, O'Sullivan B, Lazarou LP, de Castro DG, Parry S, Dodson A, Taniere P, Clark C, Butler R (2013) Improvement in the quality of molecular analysis of EGFR in non-small-cell lung cancer detected by three rounds of external quality assessment. *J Clin Pathol* 66:319–325